

PHOTOPERIOD CONTROL OF DIAPAUSE IN *DAPHNIA*. III. TWO-STIMULUS CONTROL OF LONG-DAY, SHORT-DAY INDUCTION

R. G. STROSS

*Department of Biological Sciences, State University of New York
at Albany, Albany, New York 12203*

Females of *Daphnia*, as of all Cladocera, may be reproductively polymorphic, and produce two structurally and functionally different types of eggs, one of which later enters an embryonic diapause. Expression or display of the polymorphism has been claimed to be under the control of two stimuli (Stross and Hill, 1965; 1968). One of the stimuli is an inductively short photoperiod. The second is unidentified, but its effect may be proportional to density of culture. The indicated function of the second stimulus is to trigger expression when daylength is permissively short. The hypothesis of dual stimulation is further examined in this paper.

The validity of photoperiod, or daylength, control is supported by more recent study. Comparison of populations of *Daphnia* living at 45° and 71° N, although of different species shows a latitudinal adjustment in critical photoperiod. At 45° N it is 13 hours, and at 71° N it is 22 hours of light per day at constant temperature (12° C). Photoperiod has also been implicated in the display of the reproductive polymorphism of other species of Cladocera (Parker, 1966; Shan and Frey, 1968).

The question posed in the experiments reported here is whether or not photoperiod and a density-proportional stimulus may control expression of the reproductive polymorphism in all seasonal cycles. The hypothesis of dual stimulation is based on experiments with a monocyclic strain in which expression in nature is restricted to autumn. In *Daphnia pulex* Leydig there are at least two other seasonal cycles. In the acyclic strain, the adults produce only one kind of egg, and embryonic diapause is absent. In the dicyclic strain the reproductive polymorphism may be expressed during the long daylengths of late spring as well as in short daylengths of autumn. The apparent need for both long-day and short-day induction, which is seemingly necessary to explain control by photoperiod, presents a challenge to the hypothesis of dual stimulation since no single organism is known to be both long and short-day inductive for the same polymorphism.

An attractive feature of dual stimulus control is that the observation of early investigators may be explained. Berg (1931) places the early literature into three categories or viewpoints. One view, championed by Weismann (1879), believed control was intrinsic, except for certain labile periods, and the number of generations elapsing between successive expressions of the polymorphism was believed to be under genetic control. A second group believed control to result from a variety of external stimuli associated with culture density (*e.g.* metabolites, nutritional level, *etc.*) or temperature. A third viewpoint, held by Woltereck (1911)

believed that the display resulted from an environmental induction of a pre-induced responsiveness of the organism. Woltereck (1911) identified the pre-inductive stages in the life cycle, and his embryonic stage of the mother corresponds to the most sensitive stage for induction by photoperiod (Stross and Hill, 1968). In retrospect it is possible to reinterpret intrinsic control as resulting from the then unrecognized action of daylength and extrinsic control to be the manifestation of what may be identified as the second or density-proportional stimulus.

With the recognition that photoperiod may be involved, some of the earlier descriptions of reproductive types of females (Grosvenor and Smith, 1913, Berg, 1931; 1934) have been abandoned, following Lees' (1959) treatment of the same phenomenon in an aphid. Females which are induced during embryonic or early post-natal life may produce only the short-day, or yolky, type of egg which enters an embryonic diapause. Females may also be induced later in life, and, as a result, may produce both types of eggs during their reproductive life. The short-day induced state is also reversible (Stross and Hill, 1968; Stross, 1969). Thus the distinction of female type, at least for present purposes, seems unnecessary. Also avoided in the description of a female type is a specific identification of females that produce broods of males. Males are a necessary part of the polymorphic display in some strains of *D. pulex*, although in other strains of the species and, presumably, in all strains of another species (*D. middendorffiana*) both types of embryos are produced parthenogenetically. In the bisexual strains, broods of males develop parthenogenetically and precede the shift to production of the yolky type of egg. In the one case examined by Stross and Hill (1968), the induction of males was also under the control of photoperiod, and a single female was found to be capable of producing young (both male and female) and the yolky eggs which later enter diapause. The significant feature of the reproductive polymorphism may be the shift from the production of broods of non-diapausing to the production of broods of diapausing embryos; such a designation facilitates description.

METHODS AND MATERIALS

Only minor modifications were imposed in the procedures reported earlier (Stross and Hill, 1968). The test animals, beginning as young, were cultured for 30 days under controlled photoperiods and temperatures at standardized densities. Experiments with both the dicyclic and acyclic strains of *Daphnia pulex* were carried out with the descendants of one female.

Modifications in the procedures consisted of the use of filtered lake water (Rensselaer Lake, Albany, New York) after it had been allowed to stand in pyrex carboys from one to several weeks. Light intensity in the photoperiod chambers was 450 lux. Another modification was the maintenance of constant temperature in experiments carried out at 12° C during the bi-daily census and transfer to fresh food suspensions; transfer in experiments at 19° C was carried out at room temperature (21° to 24° C). All treatments are reported as the means of four replications and were tested for statistical significance when responses were intermediate to the all-or-none.

The dicyclic strain of *D. pulex* was collected from experimental ponds at Ithaca (42° N), New York. Adults were collected in June, and young were col-

lected in November following termination of the summer period of diapause. Collections of Dr. Donald Hall, Cornell University, were examined to verify the late spring expression of the reproductive polymorphism. Collection during the winter confirmed the supposition that the population re-enters embryonic diapause in autumn.

The acyclic strain was collected from Lake Conesus (43° N), New York. This strain may be classed as operationally acyclic, since all adults in a collection made in early December 1968 were reproducing broods of the non-yolky, non-diapausing embryos. A collection (1 April, 1969) after the lake had been frozen for several months contained egg pods with the diapausing embryos, although the adults collected were producing broods of the non-yolky embryos only. The egg pod,

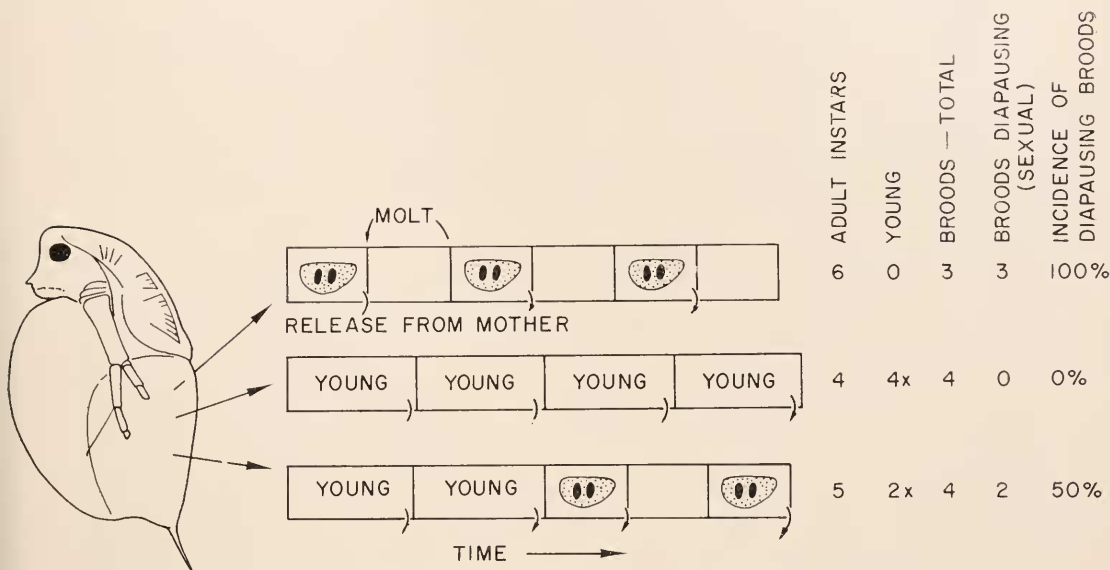


FIGURE 1. Sequence of reproductive instars in *Daphnia* and scoring procedure. The rationale for scoring broods rather than reproductive products (young and diapausing embryos) is diagrammed. See text for explanation.

or ephippium, in this strain lacks the characteristic black pigmentation, whether produced in the laboratory or lake.

Requirements for termination of the summer diapause were determined with embryos of *D. pulex* which had been collected from a small pond in Boulder, Colorado (40° N) in late August and transported to the laboratory while maintained in the natural light. In the laboratory one group was placed immediately into the experimental environments. A second group was divided, placed in sealed jars and stored in the dark at 4° or 20° C until May of the following year. The temperatures and photoperiods necessary to terminate diapause were tested in duplicates of 50 egg pods, containing 86.7 ± 4.4 (ideally 100) embryos, contained in beakers with 50 ml of lake water. The embryos, within the egg pods, were subjected to a light intensity of 1350 lux. The beakers were examined daily,

after hatching began, those in dark less frequently with the aid of a safe lamp emitting far-red light.

The scoring procedure measures the output of broods yielding diapausing embryos relative to the total output of broods (Fig. 1). At two-day intervals all released young, egg pods, and exuviae are counted in each replicate. When more than one adult is present in a vial, the number of broods yielding young is determined by a combination of (1) the number of exuviae in excess of the number of egg pods, (2) the number of barren instars predicted by the previous release of diapausing broods, and (3) the probable number of young per brood. The uncertainty is small since any given adult usually produces only one type of brood throughout the assay period, and if reproduction shifts, it usually does so only once during the 30-day assay interval.

Ideally, one may wish to compare the time interval spent in producing young or diapausing embryos. The duration of an adult instar producing diapausing embryos may, at 12° C, be only $\frac{2}{3}$ as long as an instar yielding a brood of young (Fig. 1). Moreover, a barren instar normally follows an instar which produces diapausing embryos (Stross, 1969), such that total relative duration of successive broods of diapausing embryos is approximately 1.3 times the duration of successive broods of young; at temperatures above or below 12° the ratio may be different.

A procedure, often reported in the literature, is to add young and egg pods, or their embryos, but it is completely unsatisfactory. In well fed cultures the time devoted to producing one young may be only a fraction of that spent producing an embryo that enters diapause. For example, an individual female (or group of females) may spend similar amounts of time releasing broods of young and diapaused embryos, as illustrated in Figure 1. Scored as broods, the incidence of diapausing broods is $\frac{1}{2}$ or 50%. Scored as reproductive products in which the number of young (X) is say, 20 per brood, which often is the case in well fed cultures, the incidence of diapause is 1/21 or roughly 5%, a gross underestimate of the relative time devoted to producing diapausing embryos. The objective is, of course, to describe precisely the reproductive response of cultures exposed to daylengths intermediate to the inductive and non-inductive photoperiods.

RESULTS

The dicyclic strain

In contrast to the monocylic, photoperiod control of the dicyclic strain is temperature sensitive within the range tested (13° and 19°). When cultured individually at 13° C, essentially all broods are of young at L16:D8 and all broods are of diapausing embryos at L12:D12 (Fig. 2A). The critical photoperiod, or 1:1 ratio of diapausing to non-diapausing broods, is approximately L13:D11, essentially the same as for the monocylic strain which had been collected at a similar latitude (Stross and Hill, 1968).

Sensitivity of the dicyclic strain to photoperiodic induction is also maximal during embryonic and early postnatal life. Transferred to long daylength (L16:D8) from short daylength (L11:D13) 1 to 3 days after birth, 12% of the broods were of diapausing embryos (Fig. 2A). Born and tested in long days (L16:D8).

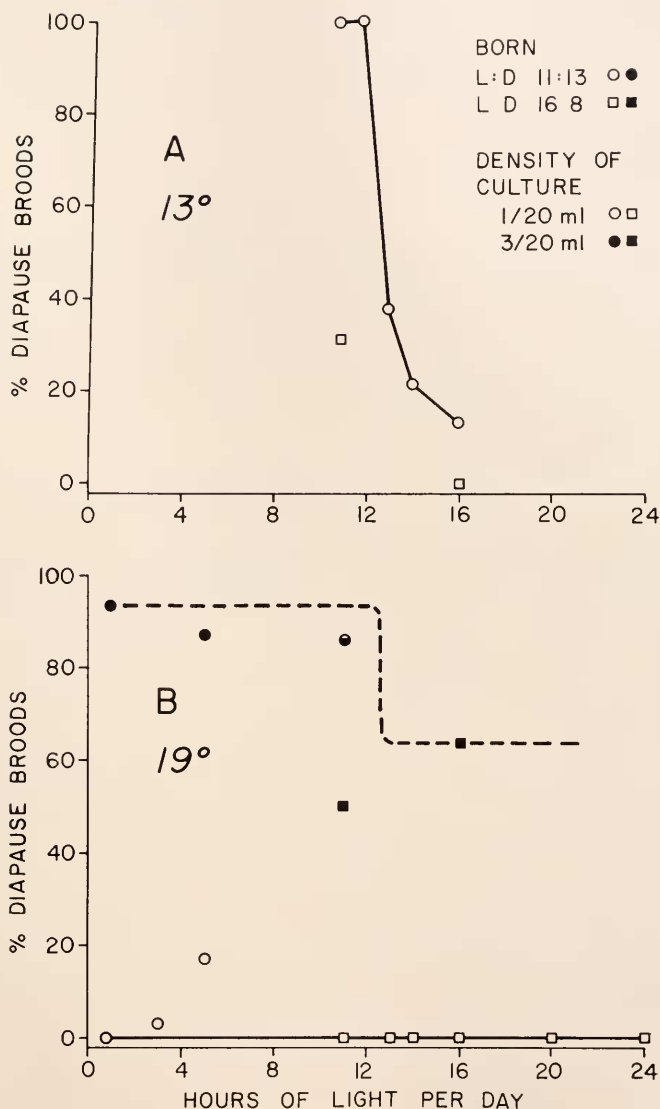


FIGURE 2. Environmental control of reproduction in the dicyclic strain. At 13° C (A) the reproductive switch to the release of diapausing embryos is short-day inductive. Mothers born in short days (open circles) are much more responsive to post-natal induction. At 19° C (B) the incidence of diapausing broods is nearly zero when the mothers are cultured individually (open symbols). When cultured at a density of 3 per 20 ml (solid symbols), the incidence is large, and larger when the mothers are born in short days (solid circles).

the adults produced only broods of young. Born in long days and tested in short, the adults produced only 31% ($P = 0.01$) diapausing broods as compared with 100% when born and tested in short days. A second test, also at 13° C, gave nearly identical values. The sharpness of the response to photoperiod when tested at a density of one adult per 20 ml and the restriction of maximum sensitivity to a limited phase of the life cycle are reasonable criteria of photoperiod control in the dicycle.

Display of the reproductive polymorphism at 19° C is primarily a function of culture density. There is essentially no photoperiodic effect when the dicyclic strain is cultured at a density of 1 per 20 ml at photoperiods ranging from L1:D23 to constant light (Fig. 2B). Only 2 of 36 females produced broods of diapausing embryos, and they were at L3:D21 and L5:D19. The apparent absence of a photoperiodic response when individual females are cultured at 19° C may be described as thermal uncoupling of photoperiod control. A similar response has been observed in insects (Paris and Jenner, 1959; Bunning and Joerrens, 1960; Lees, 1959), and could describe the results of a previous study with *Daphnia magna* (Mortimer, 1935).

The reproductive polymorphism may be displayed at 19°, however, and in long-day photoperiods. At 19° C, a water temperature comparable to that of late spring, display is controlled by a stimulus associated with density of the culture. Nearly all (93%) broods were of the diapausing embryos at a density of 3 per 20 ml, but only when the mothers were born in a short-day photoperiod (Fig. 2B). Born in a long daylength (L16:D8), and tested at the same density, the females produced a significantly smaller ($P = 0.01$) percentage of diapausing broods. The mean incidence of diapausing broods was 63.1%. The smaller proportion of diapausing broods released by mothers born in a long day-length may be the result of prenatal experience, since there was no statistical difference in the proportion of diapausing broods released in L16:D8 and in L11:D13 at 19° (Fig. 2B, solid squares). Additional tests verified the response. Therefore, it seems apparent that photoperiod continues to exert an effect at 19°, although its effect may be restricted to the prenatal or early post-natal life of the mother.

The requirement of dual stimulation was not demonstrated at 13° C, although a photoperiod and a density proportional stimulus may be functioning at 19° C. At 13° the response to short daylengths was the same (100% broods of diapausing embryos) at densities of one adult in 20, 50, and 100 ml. A density stimulus may exert an influence, however. For example, reversal of reproduction from the short to the long-day type is much slower when culture density is increased from 1 to 2 females per 20 ml.

The acyclic strain

Some of the populations free of embryonic diapause in nature may release broods of diapausing embryos in the laboratory (Berg, 1931). In this study the acyclic strain also displayed the reproductive polymorphism when cultured in the appropriate environment. At 13° C, which may represent autumn temperatures in the field, no diapause broods were produced in a long daylength (L16:D8; Fig. 3A). Culture densities were 1, 3 and 5 adults per 20 ml. In a short daylength (L11:D13), diapausing broods were produced, but only at densities of 5 and

10 adults per 20 ml (Fig. 3C). Since the total number of broods released per culture was, for practical purposes, constant at those two densities, the numbers of diapausing broods released per culture was subjected to an unpaired test of "t." The mean number released per culture at a density of 10 adults per 20 ml (10.8) was larger ($P = 0.01$) than the mean number of diapausing broods released at a density of 5 per 20 ml (2.0). The mean number of diapausing broods accounted for 73% of the total broods released at a density of 10 adults per 20 ml. Direct tests of the transformed (arc-sin) percentages are also significantly ($P = 0.01$) different.

At 19° C and short daylength, the incidence of diapausing broods was suppressed, as determined by comparison at a density of 10 adults per 20 ml (Fig. 3D). The number of diapausing broods was again larger ($P = 0.05$) at a density of 10 adults than at a density of 5 adults per 20 ml. The percentage of the total broods released as diapausing broods was 24.5% at L11:D13. There was a partial overriding of the long-day effect at the higher temperature but only at the maximum density tested, or 5 adults per 20 ml (Fig. 3B). There was also no difference in the reproductive response of adults whether born at the long or the short daylength.

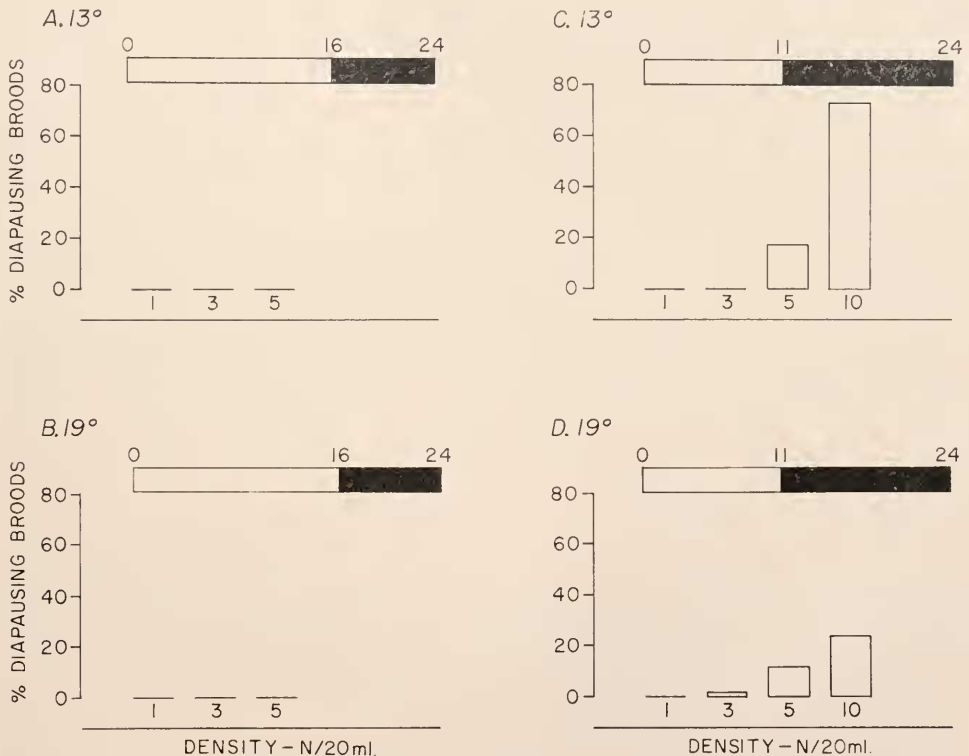


FIGURE 3. Display of the reproductive polymorphism in the acyclic strain in response to photoperiod and density. The reproductive shift occurs in short days and is transitional at a density of 5 adults per 20 ml. The shift is largest at the lower temperature.

The primary distinction of the acyclic strain seems to be its relative insensitivity to the crowding stimulus. Although individuals of the acyclic and dicyclic strains are equally large, a threshold density of 4 or 5 individuals per 20 ml is required to induce the reproductive shift in the acyclic strain. A single adult of the dicyclic strain is adequate for an essentially complete shift in reproduction when cultured in the same environmental conditions.

Male induction

In the monocyclic strain, males are short-day inductive (Stross and Hill, 1968). In the acyclic strain, males are also short-day inductive. The clone from Conesus Lake proved to be ideal for testing the response to daylength. The lack of a reproductive shift in short daylengths at low culture density permits continual observation of parthenogenesis, hence the conditions under which males are induced. At 13° C and short daylength, males were produced at all densities (1, 3, 5, and 10 adults per 20 ml; Table I). Males were produced with equal frequency to mothers born in either long or short days, and accounted for approximately 36% of the young released.

Temperature may be an important variable, both in this study and historically. At 19° C, the cultures yielded only an occasional male, or approximately 1.0% in both long and short-day photoperiods (Table I). A similar temperature influence has been observed on induction of males in the aphids (Kenten, 1955; Lees, 1959). Short daylengths acting through the food plants may also be required for induction of males in the aphid, *Megoura* (Von Dehn, 1967). Earlier reports of a temperature influence on the production of males are equivocal for *D. pulex* (Brown and Banta, 1932).

Broods of young *Daphnia* tend to be of a single sex as with another Cladoceran *Moina* (Banta, 1925). In cultures of the acyclic strain containing one adult, 13 broods were all females, 8 all males, and only 2 were of both sexes. In these cultures, the broods of males were released primarily in the second and to a lesser extent in the third brood. None of the first broods (8) contained males regardless of whether the mother was born at long or short daylengths. At densities of 3, 5 and 10 per 20 ml, males appeared in the first broods. The dicyclic strain did not produce males, nor are they required for the production of viable embryos.

Termination of summer diapause

Thermal and photic requirements for reactivation of embryos in summer diapause reveal a minimum of two-stages in a diapause development as observed in insects (Adkisson, 1965). The first or refractory phase was completed to some extent at 12° C (19.0% of viable embryos), maximally at 4° (80.0%) and not at all at 20° C. Summer diapause may have a thermal optimum slightly higher than observed for the winter diapause of the monocyclic strain. The partial effectiveness of 12° C in the former may be evidence of this. Masaki (1956) noted a higher thermal optimum in a summer diapausing moth. Some progress toward the completion of the refractory phase may occur at 20° C, although completion is unlikely. Embryos incubated for nine months at 20° C required only approxi-

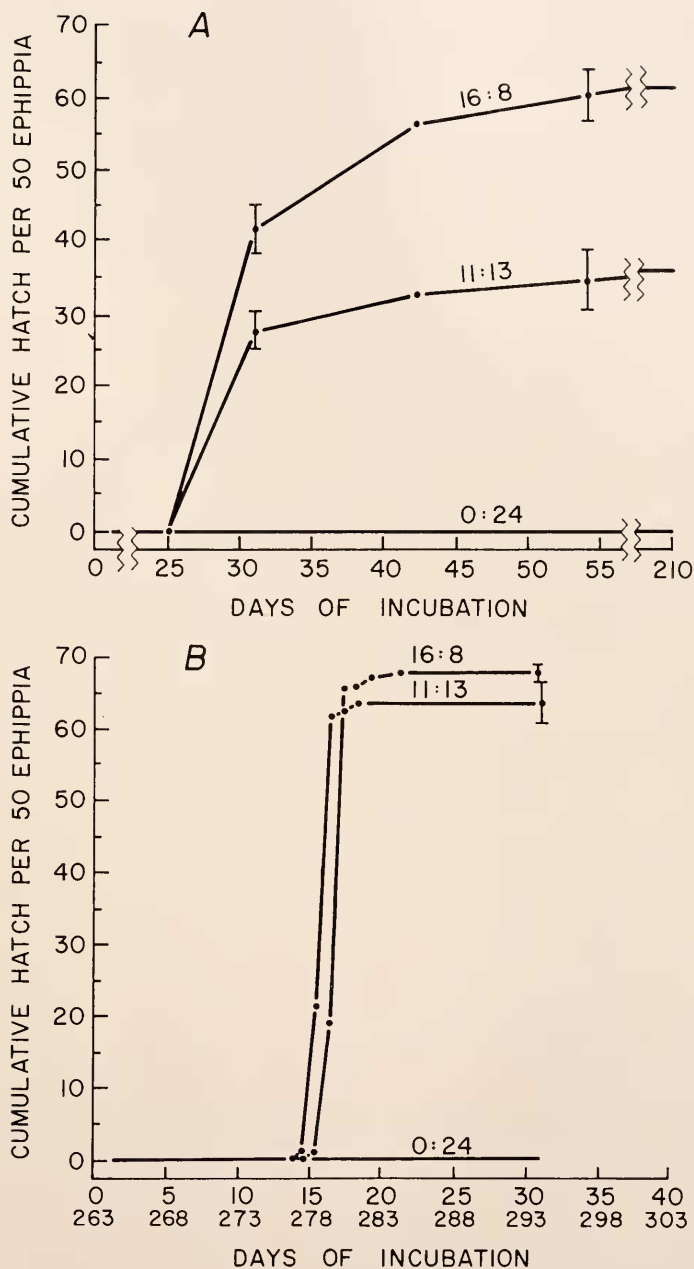


FIGURE 4. Hatching of diapaused embryos from a dicyclic strain of *D. pulex* in summer diapause. Embryos were incubated at 4° C in either the light or the dark. The influence of photoperiod (A) disappeared (B) after incubation at 4° in the dark.

mately one-half as much incubation at 4° C before becoming sensitive to the light stimulus.

The duration of the refractory interval may be only two or three weeks when incubated at 4° C. When transferred to 4° C four days after collection in August, the embryos began hatching sometime between days 25 and 30 of incubation (Fig. 4A). When the embryos were incubated for some months in the dark at the same temperature, and then exposed to light, hatching began on day 15 following transfer of the embryos to light. The 15-day interval may be essentially all post-diapause development at 4° C. Subtracting 15 days from the

TABLE I

Photoperiod, density, and temperature and the production of males in a clone from an acyclic strain of Daphnia pulex. Mothers were cultured for a period of 30 days, beginning as young as 0 to 2 days old.

		Density (Number/20 ml)				
		1	3	5	10	Totals and means
Test temp. 13°C						
Test photoperiod 16:8						
Parents born 11:13	young	80	186	256	—	522
	% males	0.0	0.0	0.0	—	0.0
Parents born 16:8	young	110	154	189	—	453
	% males	0.0	0.0	0.0	—	0.0
Test photoperiod 11:13						
Parents born 11:13	young	128	187	166	—	481
	% males	46.1	29.9	39.7	—	36.0
Parents born 16:8	young	121	162	150	39	472
	% males	43.8	26.5	38.0	41.0	35.8
Test temp. 19°C						
Test photoperiod*						
Parents born 18:8	young	377	689	571	—	1637
	% males	2.4	0.6	0.0	—	0.8
Parents born 11:13	young	346	538	436	372†	1692
	% males	0.0	1.3	0.0	1.6	0.8

* Parents born at both L16:D8 and L11:D13.

† Parents born only at long days (L16:D8).

median time of 29 days required for hatching immediately after collection may suggest the time actually required. The two weeks is approximately one-tenth the duration of the refractory interval of winter diapause in the monocyclic strain (Stross and Hill, 1968).

The brief duration of the refractory phase of diapause development is evidence that a dicyclic strain may function in nature. That is, the same population may be active in spring and in autumn after spending the summer in an embryonic diapause. It should be stressed that the above test of the refractory interval may be applicable only to diapaused embryos which have been exposed to a summer environment for an unknown interval prior to collection.

A light stimulus is required for completion of the second, or photo-sensitive phase of diapause. Moreover, the light could be demanded in the form of a long-day photoperiod. Only embryos incubated in light were activated (Fig. 4A). Also, the number of embryos which hatched in a long daylength (L16:D8) was significantly larger than the number in a short daylength (L11:D13). The fact that embryos hatched in the short photoperiod may be interpreted either as the absence of photoperiod control in a part of the population, or the interaction of an uncontrolled variable with the photoperiod control mechanism. The latter is suspected. The absolute requirement for light persisted while the photoperiod effect disappeared after incubation at 4° C in the dark for nine months (Fig. 4B). Embryos of the monocyclic strain may lose the absolute requirement for light during the course of diapause development (Stross and Hill, 1968).

DISCUSSION AND CONCLUSIONS

Three strains of *Daphnia pulex* may display a reproductive polymorphism in response to two stimuli. Each strain was selected to represent one of three seasonal patterns identified by Berg (1931). All three strains may respond to photoperiod. They seem to differ primarily in sensitivity to the second, or density proportional, stimulus. Comparison of the acyclic and dicyclic strains suggests a several-fold difference in sensitivity. Two of the strains, the monocyclic and the dicyclic, may also differ in the temperature at which photoperiod control becomes uncoupled. In this study, what is described as thermal uncoupling was evident in the dicycle strain at 19°, while in the monocyclic strain there was no evidence of it at 19° C (Stross and Hill, 1968).

The requirement for two stimuli is clearly revealed in the reproductive pattern of the dicyclic strain. At autumn temperatures (13° C and colder) the production of diapausing embryos is short-day inductive. At late spring temperatures (19° and warmer), photoperiod is not completely effective and the reproductive shift is effected by a stimulus whose effect may be density-proportional. The less extensive shift of mothers born in a long daylength at 19° C is taken as evidence that photoperiod continues to exert a partial effect at that temperature. In other words thermal uncoupling, or a loss of temperature compensation of a photoperiodic process, may permit the reproductive shift in long daylengths when the density stimulus is present. The breakdown of temperature compensation is often observed in arthropod photoperiodism (*e.g.*, Bunning and Joerrens, 1960; Paris and Jenner, 1959). Presumably the dicyclic strain may differ from the monocyclic strain (reproductive shift in autumn only) in that some mechanism, under the control of photoperiod, becomes uncoupled at a lower temperature. Although untested, the two strains could also differ in their sensitivity to the density stimulus.

Support for a simultaneous requirement of inductive photoperiod and a density-proportional stimulus is supplied by the response of the acyclic strain at 13° C. Although releasing only young in nature during the short daylengths of late autumn, the acyclic strain may be induced to release diapausing embryos in the laboratory in short-day photoperiods, but only when sufficiently crowded. The acyclic strain is apparently less sensitive to the density stimulus than is the monocyclic and dicyclic strains, and its higher threshold of sensitivity may distinguish it as Berg (1931) has concluded.

Photoperiod control of male induction was shown earlier for the monocyclic (Stross and Hill, 1968) and in this paper for the acyclic strain. In the monocyclic strain, it was found that only mothers exposed after birth to inductive photoperiods actually produced males. Embryonic induction always resulted in the production of diapausing embryos. In the acyclic strain males were produced by mothers exposed both before and after birth to an inductive photoperiod. However, since young, hence males, may not be produced in the monocycle when the mother is induced prenatally, there is no basis for comparing the two strains.

Earlier work reviewed by Banta (1939) suggested separate stimuli were responsible for inducing males and the reproductive shift, the former by an excretory product in the medium (Banta and Brown, 1929a, b) and the latter by food deprivation, although direct support for separate stimuli may be lacking. Evidence for distinct stimuli is equivocal in the present work. Adults, presumably well fed, which release broods of 15 or more young in long daylengths may be induced to release only diapausing embryos in short daylengths. Thus, food level seems to be ruled out. However, it may be argued that a density stimulus may not be required in the situation, *e.g.*, dicyclic strain at 13° C. where a single individual is sufficient to display the polymorphism. It may also be argued that photoperiod also acts indirectly through the food supply, as in male induction of the aphid, *Megoura* (Von Dehn, 1967). This seems possible, although unlikely, since the alga is cultured in a long daylength and spends a maximum of only 48 hours in the culture vessel, although usually less since most of the cells may be consumed in a time period shorter than 48 hours.

In any event there can be no argument as to the identity of the density stimulus in the situations where the intensity of display of the polymorphism is a function of density. No experiments were undertaken to distinguish between nutritional level and metabolites. Identity of the stimulus, or stimuli, remains to be determined.

Apart from the identity of the stimulus, an important question may be whether the density stimulus functions as an independent, or as an accessory stimulus to photoperiod. There is evidence to support the latter alternative. Models of photoperiod control (Bunning, 1959; Lees, 1966; Pittendrigh, 1966) agree that a photo-inducible phase of a natural rhythm lies somewhere near the beginning, or end, of the dark period of the environment; short-day induction is achieved when the inducible phase of the organism lies within the dark period of the environment. In one model (Pittendrigh, 1966) the photo-inducible phase is proposed to be coupled to the circadian oscillation. However, for the sake of illustration, one may propose that the photo-inducible phase in *Daphnia* is not coupled, and, furthermore has a natural tendency to float in the photophase of the environmental cycle, regardless of the length. Now, it is suggested that a function of the density stimulus could be either to displace a photo-inducible phase into the dark period or to couple it to a specific phase of the circadian oscillation. Evidence for both is circumstantial although from a variety of sources.

Evidence for phase displacement is seen in the shift of the critical photoperiod to a longer daylength when an arctic strain is grown in dense cultures. An increase in density was shown to shift the critical photoperiod by more than one hour (Stross, 1969). The shift is interpreted as a small but real displacement of a photo-inducible phase.

Larger shifts are suggested in the process of male induction. The vast literature describing male induction in the Cladocera, identified above as a photoperiodically controlled process, abounds with evidence of phase displacement as a result of culture density. The phenomenon of male induction may be ideally suited since males are produced in adult instars whose beginning and end could occur at no specific time relative to the light-dark cycle. (In *Moina*, for example, the first ovulation is 65 hours after birth at 20°, 38 hours at 25° C (Banta and Brown, 1929c), and presumably intermediate at intervening temperatures (Brown, 1926). In *Daphnia* the average duration of adult instars releasing young includes fractions of a day (Stross, 1969) and ovulation is fixed to molting. The second essential fact is that at minimum density, the first brood contains only females in *Moina* (Banta, 1925) and *Daphnia* (see above), while second and/or third broods may contain males. An increase in culture density results in the appearance of males in the first brood, a fact which formed the basis of the assay in later studies by Banta. That crowding may have acted by causing a phase displacement is revealed in experiments by Banta and collaborators.

Banta and Brown (1929c) identified, through manipulation, a critical time for male induction at approximately four hours prior to ovulation. Later studies (Banta and Stuart, 1932) found that with other conditions the inductive period occurred 12 to 14 hours before ovulation. Woltereck (1911) identified a similar critical period in *Daphnia* and a second period which occurred much earlier in the development of the mother; the second or earlier period is consistent with photoperiodic induction.

The action of the crowding stimulus was to effect a delay in development and release of broods. When nearly all of the first broods released by a culture were males, the delay was approximately 13 to 14 hours (Banta and Brown, 1929d). The actual development of male broods was reported to be only $\frac{1}{2}$ hour longer. Thus the delay comes during the period prior to the release of ova into the brood pouch of the mother. Although inconclusive, it is reasonable to suspect that a density stimulus had displaced a photo-sensitive phase into the dark period.

The possibility of phase displacement is also suggested by the interaction of the density stimulus and temperature. The appearance of males in first broods of *Moina* is both a function of density and temperature (Brown and Banta, 1932). In the range of 14 and 21° C, the proportion of male broods was proportional to density. Above 21° and below 30° C an increase in culture density decreased the proportion of male broods. At 30° C an increase in culture density was again effective. In other words, crowding could be effective only when the critical period for sex-determination may be shifted to occur in the dark period of the environment, and conceivably this happens when a supposed ovarian cycle is entrainably circadian (14 to 21° C) and when it could be semi-circadian (30°). The density stimulus could act through endocrine mediation similar to that visualized by Lees (1963) or directly on the phasing of oogenesis in the target organ (ovary).

While unidentified, the density stimulus could prove to be a non-stress or token stimulus. Banta and collaborators showed that it is proportional to culture volume; one animal in 7.5 ml gave a response similar to 10 animals in 75.0 ml of medium. It is apparently non-specific and a variety of aquatic invertebrates may supply the stimulus as indeed would be the case if the stimulus is as suggested by Loomis (1957, 1964). The delicacy of stimulus action may be exemplified by

an experiment of Stuart, Cooper and Coady (1933) in which the simple stoppering of the culture vessel virtually suppressed the inductive effect of the density factor on sex determination while not affecting the number of young produced.

The well known "token" effect of the photoperiod stimulus permits the photoperiodically sensitive segment of a population to develop to the diapause (resistant) stage in a non-stressing environment. The life cycle of a terrestrial arthropod may thereby be phased to a periodically harsh physical environment. An aquatic species, *Daphnia pulex* may be capable of coordinating its life cycle in response to stimuli from both its physical (photoperiod) and its biological (density-proportional stimulus) environments. Indeed the biologically derived stimulus seems capable, in different strains, of overriding and mediating the stimulus from the physical environment. Since the biological stimulus may supply the key trigger, it is reasonable to suppose that it too functions as a cue, or token stimulus. It is generally accepted that population abundance and its competitive ability with ecologically related species may depend on the size of the initial population each spring. Initial density is obviously a function of the density of diapaused individuals and the degree of synchrony in diapause termination. To suppose that "unfavorable external conditions" (Berg, 1934), or stress inducing stimuli, are cause for the reproductive shift in *Daphnia* would seem to deny a basic strategy of arthropod populations, namely, to maximize the output of diapausing individuals.

The author thanks Miss S. Anderson for technical assistance, and Dr. L. Mason for statistical advice. He is also grateful to several individuals who struggled with earlier drafts of the manuscript. Study supported by grant 299A of Joint Awards Council, State University of New York.

SUMMARY

The reproductive polymorphism in *Daphnia pulex* may be under the control of photoperiod and a second stimulus whose effect is proportional to culture density. Originally tested with a monocyclic strain, the hypothesis is extended to describe control in two other strains of *D. pulex*.

The dicyclic strain which displays the polymorphism in both long and short-day photoperiods may be only short-day inductive by usual standards. Long-day expression results when photoperiod control is thermally uncoupled. Display of the polymorphism is then under the control of culture density.

The acyclic strain, which in nature omits the reproductive polymorphism, may be induced in the laboratory. It is the acyclic strain which demonstrates the simultaneous requirement of inductively short photoperiod and the density proportional stimulus. The three types of seasonal cycles in *D. pulex* are presumed to be genetically distinct responses to the two control stimuli. The dicyclic strain may be the most sensitive to thermal uncoupling of photoperiod control, and the acyclic strain may be least sensitive to the density stimulus.

An embryonic diapause results from display of the polymorphism. The dicyclic strain may function by virtue of a much shorter refractory interval which, for the summer diapause, was found to be approximately two weeks at 4° C.

Light is an absolute requirement for termination of the diapause, and it is suggested to be more effective when presented as a long-day photoperiod.

In an attempt to relate the requirement for dual stimulation, the reproductive polymorphism of *Daphnia* is suggested to belong to a class of polymorphisms which requires external assistance in the temporal positioning of a photo-inducible phase. Expression of the polymorphism is presumed to occur when a photo-inducible phase is displaced into the dark period of the environment by a second stimulus. Several lines of evidence, which are taken from earlier studies of sex induction in the Cladocera, seem to support the idea.

LITERATURE CITED

- ADKISSON, P. L., 1965. Light-dark reactions involved in insect diapause, pp. 344-350. In: J. Aschoff, Ed., *Circadian Clocks*. North Holland Publishing Co., Amsterdam.
- BANTA, A. M., 1925. The relation between previous sexual reproduction and the production of male offspring in *Moina macrocopa*. *Amer. Natur.*, **69**: 50-61.
- BANTA, A. M., 1939. Studies on the physiology, genetics, and evolution of some Cladocera. *Carnegie Inst. Washington Publ.*, **513**: 1-285.
- BANTA, A. M., AND L. A. BROWN, 1929a. Control of sex in Cladocera I. Crowding the mothers as a means of controlling male production. *Physiol. Zool.*, **2**: 80-92.
- BANTA, A. M., AND L. A. BROWN, 1929b. Control of sex in Cladocera II. The unstable nature of the excretory products involved in male production. *Physiol. Zool.*, **2**: 93-98.
- BANTA, A. M., AND L. A. BROWN, 1929c. Control of sex in Cladocera III. Location of the critical period for the control of sex. *Proc. Nat. Acad. Science*, **15**: 71-81.
- BANTA, A. M., AND L. A. BROWN, 1929d. Control of sex in Cladocera IV. Relation between the rate of the mother's development and the sex of her young. *Physiol. Zool.*, **2**: 302-308.
- BANTA, A. M., AND C. A. STUART, 1932. The critical period for the control of sex in *Moina*. *Proc. Soc. Exp. Biol. Med.*, **29**: 1253-1255.
- BERG, K., 1931. *Studies on the Genus Daphnia* O. F. Müller: with Especial Reference to the Mode of Reproduction. Bianco Luno A/S., Copenhagen, 222 pp.
- BERG, K., 1934. Cyclic reproduction, sex determination and depression in the Cladocera. *Biol. Rev.*, **9**: 139-174.
- BROWN, L. A., 1926. Temperature characteristics for duration of an instar in Cladocerans. *J. Gen. Physiol.*, **10**: 111-119.
- BROWN, L. A., AND A. M. BANTA, 1932. Control of sex in Cladocera VII. Male production in relation to temperature. *Physiol. Zool.*, **5**: 218-229.
- BÜNNING, E., 1959. Physiological mechanism and biological importance of the endogenous diurnal periodicity in plants and animals, pp. 507-530. In: R. B. Withrow, Ed., *Photoperiodism and Related Phenomena in Plants and Animals*. AAAS Symp. #55, Washington, D. C.
- BÜNNING, E., AND G. JOERRENS, 1960. Tagesperiodische antagonistische Schwankungen der Blauviolett- und Gelbrot-Empfindlichkeit als Grundlage der photoperiodischen Diapause-Induktion bei *Pieris brassicae*. *Z. Naturforsch.*, **15b**: 205-213.
- GROSVENOR, G. H., AND G. SMITH, 1913. The life cycle of *Moina rectirostris*. *Quart. J. Microscop. Sci.*, **58**: 511-522.
- KENTEN, J., 1955. The effect of photoperiod and temperature on reproduction in *Acyrtosiphon pisum* (Harris) and on the forms produced. *Bull. Entomol. Res.*, **46**: 599-624.
- LEES, A. D., 1959. The role of photoperiod and temperature in the determination of parthenogenetic and sexual forms in the aphid *Megoura viciae* Buckton. I. The influence of these factors on apterous virginoparae and their progeny. *J. Insect Physiol.*, **3**: 92-117.
- LEES, A. D., 1963. The role of photoperiod and temperature in the determination of parthenogenetic and sexual forms in the aphid *Megoura viciae* Buckton. III. Further properties of the maternal switching mechanism in apterous aphids. *J. Insect Physiol.*, **9**: 153-164.
- LEES, A. D., 1966. Photoperiodic timing mechanisms in insects. *Nature*, **210**: 986-989.

- LOOMIS, W. F., 1957. Sexual differentiation in *Hydra*: control by carbon dioxide tension. *Science*, **126**: 735-739.
- LOOMIS, W. F., 1964. Microenvironmental control of sexual differentiation in *Hydra*. *J. Exp. Zool.*, **156**: 289-306.
- MASAKI, S., 1956. The local variation in the diapause pattern of the cabbage moth, *Barathra brassicae* Linne, with particular reference to the aestival diapause (Lepidoptera: Noctuidae). *Bull. Fac. Agr. Mie Univ.*, **13**: 29-46.
- MORTIMER, C. H., 1935. Untersuchungen über den Generationswechsel der Cladoceran. *Naturwissenschaften*, **23**: 476-480.
- PARIS, O. H., AND C. E. JENNER, 1959. Photoperiodic control of diapause in the pitcher-plant midge, *Metrionemus knabi*, pp. 601-624. In: R. B. Withrow, Ed. *Photoperiodism and Related Phenomena in Plants and Animals*. AAAS Publ. #55, Washington, D. C.
- PARKER, R. A., 1966. The influence of photoperiod on reproduction and molting of *Daphnia schodleri* Sars. *Physiol. Zool.*, **39**: 266-279.
- PITTENDRIGH, C. S., 1966. The circadian oscillation in *Drosophila pseudoobscura* pupae: a model for the photoperiodic clock. *Z Pflanzenphysiol.*, **54**: 275-307.
- SHAN, R., AND D. G. FREY, 1968. Induced interbreeding between two stocks of a chydorid Cladoceran. *Bioscience*, **18**: 203-205.
- STROSS, R. G., 1969. Photoperiod control of diapause in *Daphnia* II. Induction of winter diapause in the arctic. *Biol. Bull.*, **136**: 264-273.
- STROSS, R. G., AND J. C. HILL, 1965. Diapause induction in *Daphnia* requires two stimuli. *Science*, **150**: 1462-1464.
- STROSS, R. G., AND J. C. HILL, 1968. Photoperiod control of winter diapause in the fresh-water crustacean, *Daphnia*. *Biol. Bull.*, **134**: 176-198.
- STUART, C. A., H. J. COOPER AND H. COADY, 1933. Carbon dioxide as a sex determining factor in *Moina macrocopa*. *J. Exp. Biol.*, **10**: 47-58.
- VON DEHN, M., 1967. Über den Photoperiodismus heterogoner Aphiden. Sur Frage der Direkten oder Indirekten Wirkung der Tageslänge. *J. Insect Physiol.*, **13**: 595-612.
- WEISMANN, A., 1879. Beiträge zur Naturgeschichte der Daphnoiden. *Z. Wiss. Zool.*, **33**: 55-270.
- WOLTERECK, R., 1911. Über Veränderung der Sexualität bei Dapnoiden. *Int. Rev. Gesamten Hydrobiolog.* **4**: 91-128.